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Antibodies to the Trypsin Cleavage Peptide VP8* Neutralize Rotavirus by Inhibiting Binding of Virions to Target Cells in Culture

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Two distinct patterns of neutralization were identified by comparing the neutralization curves of monoclonal antibodies (MAbs) directed at the two surface proteins, VP4 and VP7, of rhesus rotavirus. VP7-specific MAbs were able to neutralize virus efficiently, and slight increases in antibody concentration resulted in a sharp decline in infectivity. On the other hand, MAbs to VP4 proved much less efficient at neutralizing rhesus rotavirus, and the fraction of infectious virus decreased gradually throughout a wide range of antibody concentrations. MAbs directed at VP8*, the smaller trypsin cleavage fragment of VP4, were shown to efficiently prevent binding of radiolabeled virions to MA104 cell monolayers, to an extent and at concentrations comparable to those required for neutralization of infectivity. Conversely, MAbs recognizing VP7 or the larger VP4 trypsin cleavage product, VP5*, showed little or no inhibitory effect on virus binding to cells. All MAbs studied were able to neutralize rotavirus that was already bound to the surface of cells. The MAbs directed at VP8*, but not those recognizing VP5* or VP7, were shown to mediate release of radiolabeled virus from the surface of the cells. With MAbs directed at VP7, papain digestion of virus-bound antibody molecules led to an almost complete recovery of infectivity. Neutralization could be fully restored by incubation of virus-Fab complexes with anti-mouse immunoglobulin G antiserum. Neutralization with MAbs directed at VP8* proved insensitive to digestion with papain as well as to the addition of anti-immunoglobulin antibodies.

Rotavirus is a major cause of acute gastroenteritis in humans as well as many animal species (22). In recent years, considerable efforts have been made to understand the antigenic properties of the virus as well as the immunological aspects of infection, in the hope that this effort would eventually lead to the development of an effective vaccine (26).

Rotavirus possesses a relatively complex icosahedral structure with an inner core containing 11 segments of genomic double-stranded RNA surrounded by two distinct protein layers that compose the viral capsid (11). Most of the outer capsid is made of a 37-kDa glycoprotein known as VP7, which is encoded by gene 7, 8, or 9 depending on the virus strain (11). VP7 is primarily responsible for the serotype specificity of isolates. The second surface component is the nonglycosylated protein, VP4. This protein is the product of gene 4, is the viral hemagglutinin, and appears to be responsible for restriction of growth in tissue culture and virulence in experimental animals (15, 31). According to high-resolution cryoelectron microscopy studies (37, 49), VP4 is present on the virion in the form of spikes passing through and extending from the virion surface layer for about 12.0 nm. Upon moderate digestion with trypsin, the 86.5-kDa VP4 molecule yields two smaller polypeptides of 65 and 28 kDa, termed VP5* and VP8*, respectively (11). Both tryptic fragments remain associated with the virion after digestion. Proteolytic cleavage, which is also believed to occur in the intestinal lumen during infection, strongly enhances rotavirus infectivity in tissue culture, probably by enhancing virus penetration of the cell (12, 13, 21).

Both rotavirus surface proteins have been proved to be immunogenic under experimental conditions as well as during natural infection (41, 44), and antibodies to both VP4 and VP7 have been used effectively to prevent virus replication in cell cultures and to protect laboratory animals passively from challenge with infectious virus (18, 26, 27, 33). By competition binding analysis with neutralizing monoclonal antibodies (MAbs), at least one neutralization domain has been identified on VP7 and at least two have been identified on VP4, each of these domains comprising several interrelated epitopes (8, 42, 45).

In view of the structural and antigenic complexity of rotavirus, it is likely that antibodies directed to different sites on the virus surface would mediate neutralization of infectivity by a variety of different mechanisms, but very little information on this issue has been reported so far (13, 26, 28, 39). In the present study, we examined a library of neutralizing MAbs directed at two distinct epitopes on VP7 and both cleavage products of VP4. Neutralization curves were obtained for each antibody in a plaque reduction assay with high concentrations of virus in suspension as well as virus previously bound to the surface of target cells. The ability of MAbs to interfere with binding or to mediate elution of prebound radiolabeled virus from the cell receptor was also investigated. Our studies indicate that antibodies directed at the VP8* fragment appear to inhibit binding of virus to cells in vitro and that this inhibition is responsible for their neutralization activity.

MATERIALS AND METHODS

Cells and virus. MA104 cells were propagated in medium 199 (M199) (Irvine Scientific, Santa Ana, Calif.) supple-

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mented with 7.5% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), 2 mM glutamine, 100 IU of penicillin G per ml, and 100 μg of streptomycin per ml at 37°C in a 5% CO_2 atmosphere. For neutralization experiments, a single stock of rhesus rotavirus (RRV) was prepared in MA104 cell monolayers grown in 175-cm^2 flasks. Prior to infection, cells were washed twice with serum-free M199 and virus was activated with trypsin (type IX; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 5 μg/ml at 37°C for 1 h. After adsorption of the virus (0.05 PFU per cell) for 90 min at room temperature, cells were refed with 20 ml of fresh medium containing 0.5 μg of trypsin per ml and incubated at 37°C for 2 days. Monolayers were freeze-thawed twice and fluorocarbon extracted; the resulting virus suspension was filtered through 0.45-μm-pore-size filters to prevent large viral aggregates and frozen at −70°C in small aliquots.

MAb purification. The RRV anti-VP4 and anti-VP7 MAbs used in this study have been described previously (2, 42). Ascites fluids were prepared in rotavirus antibody-negative BALB/c mice injected intraperitoneally with 2×10^6 hybridoma cells from exponential-phase cultures; before injection, cells were washed twice with serum-free Dulbecco minimal essential medium. Peritoneal fluid was collected after 7 to 11 days and filtered through 0.45-μm-pore-size filters. Antibodies belonging to the immunoglobulin G (IgG) classes were affinity purified with a 2-ml protein G-Sepharose column (Pharmacia, Inc., Piscataway, N.J.) equilibrated in 10 mM phosphate buffer (pH 7.0) and with 20 mM glycine (pH 2.8) as the elution buffer. Eluted immunoglobulins were dialyzed against phosphate-buffered saline (PBS), concentrated with Centricon 30 microconcentrators (Amicon Corp., Danvers, Mass.), and stored frozen at −70°C until use. Protein concentration was determined by the Bradford method (3). IgM antibodies M14 and 57.8 were purified by high-pressure liquid chromatography with a 4.5- by 250-mm Bakerbond MAb column (J. T. Baker Chemical Co., Phillipsburg, N.J.) with a linear KH_2PO_4 gradient from 10 to 250 mM at pH 7.0 (flow rate, 0.5 ml/min). Concentrated antibodies were stored at −70°C in the presence of 50% glycerol.

Neutralization assays. Two different neutralization assays were used. In the first, trypsin-activated RRV was diluted with 1% bovine serum albumin (BSA) in M199 to give a concentration of approximately 10^8 PFU/ml. Aliquots of the viral suspension were then reacted with each MAb through a wide range of antibody concentrations. Neutralization took place for 2 h at 37°C and overnight at 4°C; each reaction mixture was then serially diluted and plated onto MA104 cells in six-well plates in duplicate. After 90 min of adsorption at room temperature, monolayers were washed three times and overlaid with M199 containing 0.5 μg of trypsin per ml and 0.55% agarose (SeaKem; FMC BioProducts, Rockland, Maine). After incubation at 37°C for 3 days in a CO_2 atmosphere, plaques were stained with neutral red and counted. Data are expressed as the percentage of nonneutralized virus compared with control wells infected with virus in the absence of antibody. Neutralization was considered significant when there was a 60% or greater reduction in plaque number. In the second neutralization assay, monolayers were inoculated with different virus dilutions and incubated for 90 min in an ice bath. Previous studies had demonstrated that rotavirus binds to but does not enter MA104 cells at 4°C (14a). Unadsorbed virus was washed out with ice-cold medium, and monolayers were subsequently incubated with serial dilutions of MAb at 0°C for 90 min. After extensive washing, cells were overlaid with M199-agarose as described above. Neutralization assays were run in triplicate wells, and the results shown represent averages of number of plaques counted. All neutralization experiments were done at least twice.

Preparation and purification of radiolabeled RRV. Monolayers of MA104 cells in 850-cm^2 roller bottles were infected with 10 ml of a trypsin-activated RRV suspension at a multiplicity of infection of 5. After 1 h of adsorption at 37°C, cells were washed twice with Dulbecco PBS and refed with 10 ml of methionine-free minimal essential medium. At 4 h postinfection, medium was removed and fresh medium (10 ml) containing 100 μCi of [^{35}S]methionine (Tran^{35}S-label; ICN Radiochemicals, Irvine, Calif.) per ml was added. After 14 h of incubation at 37°C, cells were disrupted by freeze-thawing and extracted twice with fluorocarbon. Virus was pelleted through a 30% sucrose cushion in PBS in a Beckman SW40 rotor at 33,000 rpm for 90 min at 4°C and resuspended in 6 ml of 10 mM Tris–100 mM NaCl–2 mM $CaCl_2$ (TNC) at pH 7.4, containing CsCl to give a density of 1.37 g/ml. Isopycnic banding was performed with a Beckman SW40 rotor at 33,000 rpm for 20 h at 12°C. The band corresponding to the double-shelled (ds) particles was collected by puncturing the tube and dialyzed against TNC. Purified virus was stored at 4°C and used within 10 days.

Virus binding to MA104 cells. ^{35}S-labeled virions were treated with 5 μg of trypsin per ml at 37°C for 1 h and appropriately diluted in TNC containing 2% BSA before the assay. Aliquots of 10 μl were incubated with equal volumes of MAb solutions at the indicated concentrations and incubated at 37°C for 2 h and at 4°C overnight. Virus-antibody mixtures were diluted up to 400 μl with ice-cold TNC–1% BSA, and 200 μl of each (9×10^6 PFU, 0.04 pCi/PFU corresponding to approximately 10,000 cpm) was inoculated in duplicate onto MA104 cell monolayers in 24-well plates, containing approximately 10^5 cells previously washed with TNC. Virus adsorption was allowed to occur for 90 min in an ice bath with rocking. The supernatant was collected, and monolayers were washed three times with cold buffer; cells were then lysed with 2% sodium dodecyl sulfate (SDS). Supernatants and cell lysates were mixed with 10 ml of scintillation fluid, and the radioactivity was measured in a scintillation counter. The extent of virus binding for each sample was expressed as [cpm in the cell lysate/(cpm in the cell lysate + cpm in the supernatant)] × 100, after subtraction of the background (cpm is counts per minute). A 60% or greater reduction in binding was considered significant.

Detachment of preadsorbed RRV from MA104 cells. Approximately 10,000 cpm of trypsin-activated ^{35}S-labeled RRV in TNC-BSA were allowed to bind to MA104 cell monolayers in 24-well plates at 0°C for 90 min. Monolayers were then washed three times with cold buffer to remove the unbound virus, and 200 μl of MAb solution in TNC-BSA at selected concentrations was added to each well in duplicate. Incubation was continued for 60 min in an ice bath, and monolayers were washed again. Cells were then lysed as described above and counted for residual radioactivity. For normalization of data, counts in the cell lysate and counts in the supernatant after incubation with antibody were analyzed as described above. Results are expressed as percentage of virus remaining bound to the cells compared with monolayers incubated in the absence of antibody.

Papain digestion of MAbs bound to RRV. Approximately 10^7 PFU of trypsin-activated RRV were incubated with selected MAbs at a final concentration of 10 μg/ml in 0.1 ml of M199 containing 1% BSA for 2 h at 37°C and overnight at 4°C. Aliquots of virus-antibody complexes were then treated with 50 μg of papain (Sigma) per ml for 3 h at room

temperature in the presence of 10 mM cysteine and 1 mM EDTA. The reaction was terminated by the addition of iodoacetamide (Sigma) to 1.5 mM. Virus-Fab complexes were incubated with a 1:20 dilution of goat anti-mouse IgG (Sigma) for 1 h at room temperature. The goat antimouse reagent was shown not to contain detectable neutralizing antibody to RRV in this assay (data not shown). Samples of the treated virus were set aside after each procedure and assayed in duplicate for infectivity on MA104 cells in 96-well plates. After 2 h of adsorption at 37°C, the virus inoculum was removed and monolayers were washed three times with M199. At 20 h postinfection, cells were fixed with methanol and stained by an immunocytochemical method with a hyperimmune guinea pig antiserum to rotavirus as previously described (42). Rotavirus-infected cells were counted, and data are expressed as described above for plaque reduction assays.

RESULTS

Neutralization of RRV by VP7 and VP4 MAbs. Each of the four neutralizing MAbs to VP7 reduced RRV infectivity substantially after prolonged incubation with concentrated (10^8-PFU/ml) preparations of virus (Fig. 1A). For the different MAbs, a 60% reduction in the number of plaques first occurred at antibody concentrations ranging between 0.4 and 2 μg/ml; afterwards, relatively small increases in MAb concentration resulted in a sharp (3 to 4 $\log_{10}$) decline of viral infectivity (Fig. 1A). Two control nonneutralizing MAbs, one directed at VP7 (MAb 60) and the other at the nonstructural protein NS35 (MAb 191), had no significant effect even at concentrations higher than 1 mg/ml (data not shown). In the same assay and at comparable concentrations, MAbs directed to VP4 showed substantially less neutralizing activity when compared with VP7 antibodies (Fig. 1B). Maximum reductions of infectivity were between 91 and 98% and occurred at antibody concentrations of about 1 mg/ml. Neutralization titration curves for the VP4 MAbs showed a gradual decrease of infectivity over a 3-log range of antibody concentration rather than the steep decline seen with VP7 antibodies. A 60% reduction in plaque number first occurred at MAb concentrations in the 0.2- to 6-μg/ml range, similar to the range for VP7 antibodies (Fig. 1).

Inhibition of ds RRV binding to MA104 cells by MAbs. ^{35}S-labeled ds RRV particles were incubated with each MAb over a wide range of antibody concentrations, and the ability of each metabolically labeled viral sample to bind to MA104 cell monolayers was assayed. Under the conditions used in these experiments, 15 to 20% of the input radioactivity was found to remain associated with the cell monolayers in the absence of antibody. As a control for specificity of binding, purified single-shelled particles collected during preparation of ds RRV were also assayed under the same conditions, and less than 1% of the radiolabeled single-shelled virus was recovered in the cell lysate after washing. The results of the binding experiments are summarized in Fig. 2. MAbs recognizing the VP8* amino-terminal trypsin fragment of VP4 (23, 1A9, 7A12, M14) inhibited binding to cells to an extent and at antibody concentrations comparable to those observed in the neutralization experiments (Fig. 2B). MAb 2G4, which recognizes the VP5* region of the same protein, was much less effective, blocking RRV binding by only 40% when used at a concentration of about 200 μg/ml. This concentration of 2G4 is over 10 times greater than is needed to obtain a corresponding decrease in viral infectivity. No inhibition of binding was exhibited by the nonneutralizing VP7 MAb 60 and by MAb 191 directed at NS35 (Fig. 2B). Two of the neutralizing antibodies directed to VP7 (MAbs 159 and 5H3) did not inhibit viral binding at all (Fig. 2A). A partial decrease of cell-bound radioactivity was induced by the two other neutralizing VP7 MAbs, 4F8 and 57.8. However, as was the case with MAb 2G4, the amount of antibody required to reduce virus-cell binding with 4F8 and 57.8 (Fig. 2A) was at least 100-fold more than that shown to mediate neutralization of RRV infectivity.

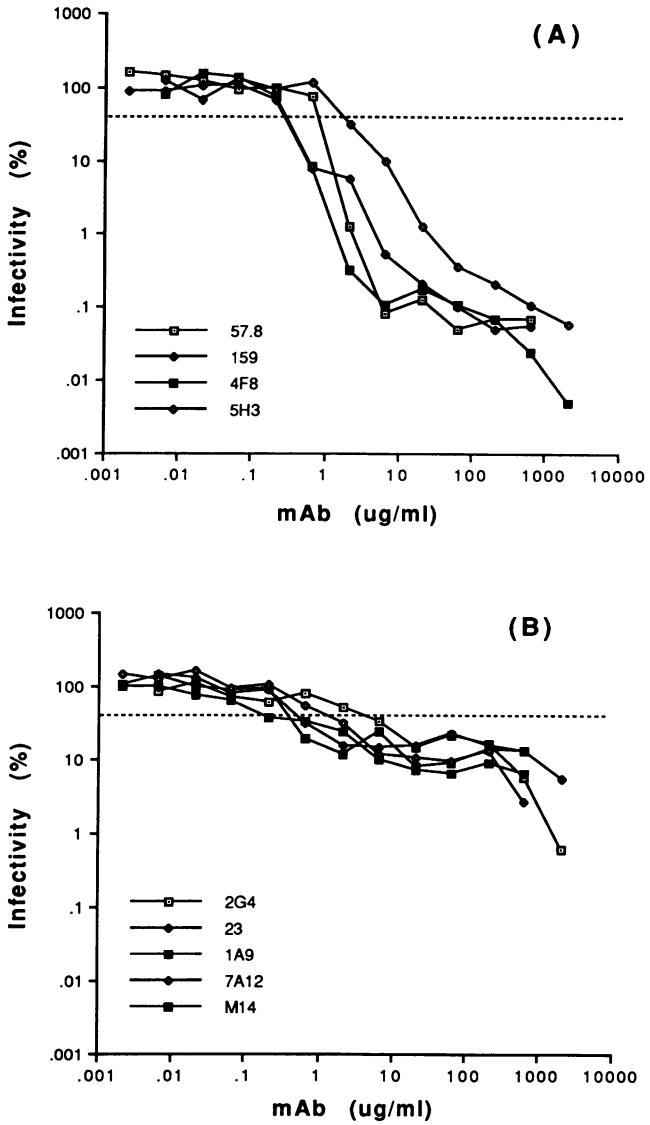

FIG. 1. Neutralization of RRV infectivity by MAbs directed at VP7 (A) and VP4 (B). Trypsin-treated RRV (10^8 PFU/ml) was mixed with purified MAbs at different concentrations, and incubation was done at 37°C for 2 h and at 4°C overnight. Virus-antibody mixtures were diluted and inoculated onto MA104 cell monolayers in six-well plates in duplicate. After adsorption (2 h at room temperature, with rocking), cells were washed three times with M199 and overlaid with M199 containing 0.55% agarose and trypsin at a concentration of 0.5 μg/ml. At 3 days postinfection, monolayers were stained with neutral red and viral plaques were counted. Data are expressed as percent residual infectivity after neutralization compared with virus incubated in the absence of antibody. For details, see Materials and Methods. Dotted line indicates 60% infectivity reduction.

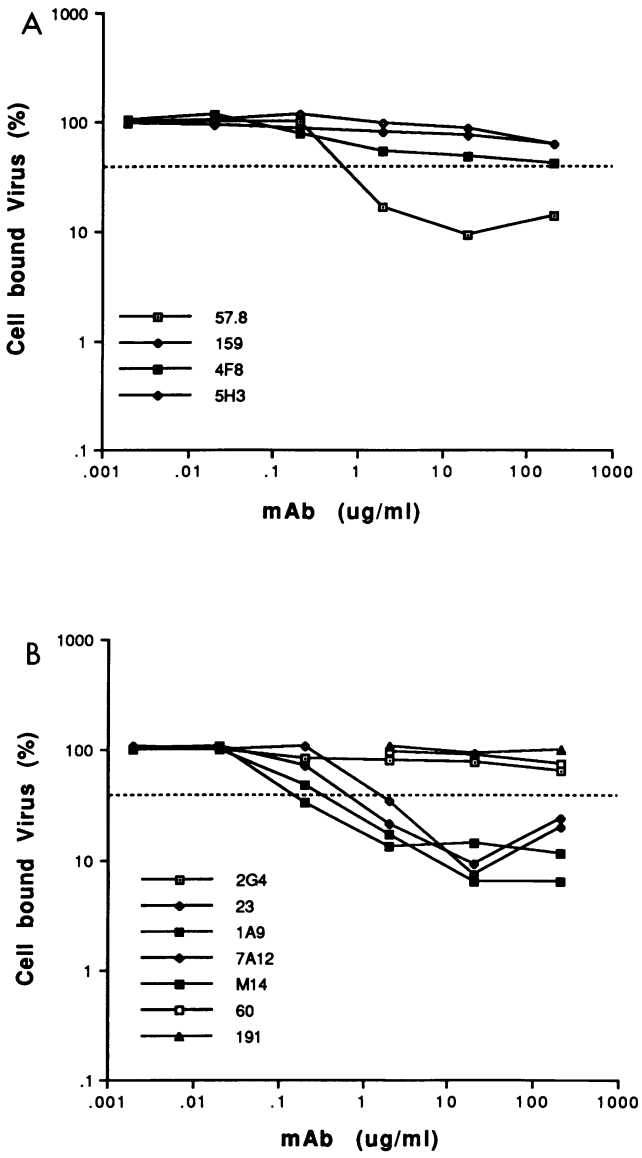

FIG. 2. Inhibition of ds RRV binding to MA104 cell monolayers by MAbs directed at VP7 (A) and VP4 (B). Approximately 10,000 cpm of trypsin-activated purified ds RRV (specific activity, 0.04 pCi/PFU) metabolically labeled with [^{35}S]methionine were mixed with serial dilutions of purified MAbs. After incubation at 37°C for 2 h and at 4°C overnight, virus-antibody mixtures were inoculated onto MA104 cell monolayers in 24-well plates (approximately 10^5 cells per well), and incubation was done at 0°C for 2 h. Supernatants were aspirated and set apart; monolayers were washed five times with PBS, and cells were lysed with 2% SDS. Supernatants and cell lysates were dissolved in scintillation medium and counted for radioactivity. Data are expressed as the percentage of cell-bound radioactivity after neutralization (compared with control cultures inoculated with virus not treated with antibody), by antibody concentration. For details, see Materials and Methods. Dotted line indicates 60% reduction in binding of labeled virus.

Recovery of infectivity by neutralized RRV after treatment with papain. Four MAbs directed at VP4 (2G4, 23, 1A9, 7A12) and three directed at VP7 (159, 4F8, 5H3) were used to neutralize RRV at concentrations yielding a reduction of virus infectivity between approximately 5- and 10,000-fold, depending on the MAb. After incubation, the virus-antibody complexes were treated with papain to generate monovalent Fab fragments. For the VP7 MAbs, this treatment led to a striking reversal of neutralization that ranged between 20- and 300-fold (Table 1). Papain treatment induced a slight recovery of infectivity by the virus neutralized with MAb 2G4, directed at the VP5* fragment of VP4. In contrast to the VP7 MAbs, neutralization by the three MAbs (23, 1A9, 7A12) recognizing the VP8* fragment proved resistant to papain digestion (Table 1). In all cases in which papain digestion had an effect, subsequent incubation of the virus-Fab complexes with a large excess of anti-mouse IgG antibodies fully restored neutralization (Table 1), indicating that the enzymatic treatment had not affected the binding ability of the cleaved antibody molecules. Interestingly, the neutralizing ability of the MAb 2G4 Fabs complexed with anti-mouse IgGs greatly exceeded that shown by the native form of this MAb. Neutralization by Fab fragments derived from the three MAbs directed at VP8* was virtually unaffected by the addition of anti-IgG antibodies.

TABLE 1. Effect of papain on RRV neutralization by MAbs

MAb tested	Residual infectivity after treatment with[a]:		
	MAb alone	MAb and papain	MAb, papain, and anti-IgG
2G4	18.2	40.4	0.46
23	9.1	27.3	14.1
1A9	9.2	16.2	8
7A12	6.3	14.1	11.1
159	0.052	5.8	0.012
4F8	0.012	38.4	0.02
5H3	1.5	32.3	0.03
No MAb	100	78.8	49.5

[a] Approximately 10^7 PFU of trypsin-activated RRV were incubated with indicated purified MAbs (20 μg/ml) at 37°C for 2 h and at 0°C overnight. Samples were incubated with 50 μg of papain per ml at room temperature for 3 h in the presence of 10 mM cysteine and 1 mM EDTA. The reaction was terminated with iodoacetamide (1.5 mM). Aliquots of the virion-Fab complexes were then incubated with a 1:20 dilution of goat anti-mouse IgG (Sigma) at room temperature for 1 h. Samples after each treatment were inoculated onto MA104 cell monolayers in 96-well plates. After 20 h of incubation at 37°C, cells were fixed and stained by an immunoperoxidase method. Wells corresponding to virus not subjected to any treatment had an average of 198 stained cells. Wells infected with RRV treated with papain in the absence of MAb showed an average of 156 foci. Data express residual infectivity after each treatment as percentage of the value obtained with control wells infected with RRV in the absence of any treatment.

Neutralization of RRV bound to MA104 cells. To explore the possibility that some MAbs might neutralize RRV that had already been bound to cells, we incubated MA104 cell monolayers with different amounts of virus at low temperature to allow viral adsorption but not cell entry. After washing, the prebound virus was exposed to selected MAbs and the residual infectivity on the monolayer was monitored by the development of plaques. Each MAb to VP7 reduced cell-bound infectivity almost as efficiently as it did in the neutralization experiments done with virus in suspension (Table 2). Of the five MAbs to VP4, MAbs 7A12 and 1A9 (VP8*) also proved to be highly effective at neutralizing cell-bound RRV (Table 2). MAbs 23 and M14, both recognizing VP8*, as well as MAb 2G4, which is directed at VP5*, had somewhat less neutralizing activity against prebound virus than against unbound virus. At maximum concentration, MAbs 23, M14, and 2G4 reduced the number of plaques by 78, 72, and 82%, respectively, compared with 92, 92, and

TABLE 2. Neutralization of RRV prebound to MA104 cell monolayers with MAbs directed at viral proteins[a]

Amt of MAb (μg/ml)[a]	% Infectivity								
	VP5* (2G4)	VP8*				VP7			
		23	1A9	7A12	M14	57.8	159	4F8	5H3
2,000	**17.8**	**21.9**	**7.5**	**16.9**	**27.8**	**0.09**	**0.05**	**0.04**	**0.34**
200	**32.9**	**30.1**	**8.7**	**19.1**	**32.2**	**0.17**	**0.07**	**0.08**	**0.55**
20	54.8	68.5	**22**	**26.7**	50.4	2	**0.42**	**1.3**	**2.6**
2	49.3	86.3	**32.2**	42.6	64.1	**22.6**	14.1	**10.9**	**25.6**
0.2	87.1	97.1	122	92.8	101.4	87.1	75.6	66.2	67.9
0.02	101.2	99.8	126.1	104.3	96.7	139	104.8	103	83.3

[a] Serial dilutions of trypsin-treated RRV were inoculated onto MA104 cell monolayers in six-well plates. After 90 min of incubation at 0°C, unbound virus was removed and cells were washed two times. M199 containing purified MAbs at the indicated concentrations was then added, and incubation was continued at 0°C for 60 min. After being washed with M199 four times, cells were overlaid with medium supplemented with 0.55% agarose and 0.5 μg of trypsin per ml. Monolayers were incubated for 3 days at 37°C, and then cells were stained with neutral red and viral plaques were counted. Control wells contained an average of 29.8 plaques. Data express the residual infectivity after incubation with MAbs as percentage of the value obtained with infected control cells incubated in the absence of antibody. Numbers in bold type represent a significant reduction (60%) in infectivity.

99%, respectively, in the preceding neutralization test done in solution.

Removal of ds RRV particles from MA104 cells with MAbs. To determine whether MAbs could detach prebound rotavirus from cells, we allowed purified ds RRV particles labeled with [^{35}S]methionine to adsorb to MA104 cells for 90 min at low temperature. After removal of unbound virus, monolayers were further incubated for 60 min in the presence of selected MAbs in an ice bath, and the radioactivity released into the medium as well as that remaining associated with the cells was counted. In the absence of antibody during incubation, approximately 10% of the bound radioactivity was spontaneously released from the cells, and similar figures were obtained after incubation with nonneutralizing antibodies 60 and 191 (Table 3). No further release of virions was induced by the neutralizing VP7 antibodies 159, 5H3, and 57.8 (Table 3). Incubation with high (200 μg/ml) concentrations of MAb 4F8 (VP7) was associated with the elution of 70% of the bound radioactivity into the medium. The VP5* MAb, 2G4, did not appear to induce the release of radiolabeled virus from the cells. Of the four MAbs directed to the VP8* region of VP4, MAb 1A9 mediated detachment from the cells of up to 84% of the total counts per minute and showed its activity over a range of concentration comparable to that previously shown to be active in neutralization experiments with cell-bound virus (Table 2). The VP8* MAbs 23 and 7A12 caused release of radioactive material into the cell supernatant to an extent only slightly less than 1A9. The release of labeled virus with MAbs 23 and 7A12 occurred at concentrations that closely approximated the concentrations observed to be active in the neutralization experiments with virus prebound to the cells.

DISCUSSION

Neutralization of viral infectivity by antibodies directed at virion proteins may occur through a variety of different mechanisms (7). Studies done with poliovirus, influenza virus, and adenovirus (4, 34, 46, 48) indicate that in some instances viral neutralization does not necessarily involve a true alteration of the intrinsic infectivity of the virus, since it can also occur via antibody-mediated aggregation of virions. Similar findings have been recently confirmed and extended by Mosser and colleagues (30), who analyzed a total of 47 distinct MAbs to poliovirus and rhinovirus. In this study, however, the authors suggest that "potent neutralizing" MAbs do not mediate their effect by aggregating virus but rather act via bivalent binding to epitopes located in two adjacent pentamers of the virus capsid. This bivalent binding appears to stabilize the poliovirus capsid itself and impair the subsequent unfolding of virion proteins. This hypothesis is consistent with earlier studies by Mandel (25), Icenogle et al. (19), and Emini and co-workers (10), who showed that bivalent binding of neutralizing MAbs to poliovirus can

TABLE 3. Elution of purified ds RRV from MA104 cell monolayers by MAbs directed at indicated viral proteins[a]

Amt of MAb (μg/ml)	% Radioactivity										
	VP5* (2G4)	VP8*				VP7					NS35 (191[b])
		23	1A9	7A12	M14	57.8	159	4F8	5H3	60[b]	
200	77.5	**37.2**	**16.1**	**38.7**	ND[c]	60.9	66.5	**30.7**	88.1	80.1	100.7
20	78.6	44.8	**32.2**	**39.2**	47.7	83.4	75	59	90.7	83.5	92.3
2	88.9	79.6	59.9	75.3	98.6	93.4	78.4	56.3	97.4	95.4	103.1
0.2	90.1	100.1	97.7	93.8	85.1	99.1	85.2	88	100.1	101.3	97.4
0.02	101.2	100.8	101	97.6	100.4	101.5	95	105.1	99.2	ND	ND

[a] 10,000 cpm of trypsin-activated ds RRV (0.04 pCi/PFU) metabolically labeled with [^{35}S]methionine were inoculated onto MA104 cell monolayers in 24-well plates. After 90 min of incubation at 0°C, unbound virions were removed and monolayers were washed three times. M199 containing purified antibodies at the indicated concentrations was added to each well, and incubation was continued at 0°C for 60 min. Supernatants were set apart, and cells were washed three times with ice-cold PBS. Cells were lysed with 2% SDS. Supernatants and cell lysates were dissolved into scintillation fluid and counted for radioactivity. Data express residual radioactivity associated with monolayers after incubation with MAbs as the percentage of the value obtained with control wells incubated in the absence of antibody. Numbers in bold type represent a significant reduction in cell-associated radioactivity (<40%).

[b] Nonneutralizing MAbs.

[c] ND, Not determined.

induce a shift in the isoelectric point (pI) of virions and that in situ digestion of the antibody molecules to monovalent Fab fragments restored both infectivity of the virus and its original pI. This conformation-altering pathway to neutralization has also been proposed for other viruses, such as adenovirus (47), influenza virus (35), and bovine enterovirus (5).

A still-debated question is whether or not inhibition of virus binding to the host cell is to be regarded as an additional mechanism of action of neutralizing antibodies. Data in support of this have been reported (9, 13, 16, 23, 38), but in several cases the quantitative relationship between neutralization and inhibition of cell attachment was not clearly addressed. Conversely, in many instances, antibody-neutralized viruses have been shown to bind efficiently to cells (9, 14, 20, 29, 43, 48). To further complicate matters, in some cases virus binding to and infection of cells can be specifically mediated by antibody. In such cases, the virus-antibody complex usually binds to the Fc receptor rather than the viral receptor (17, 40).

We have begun to investigate the mechanisms by which selected MAbs directed at the two rotavirus surface proteins, VP4 and VP7, neutralize virus. Of note, prior studies have clearly demonstrated that certain MAbs directed at VP7 and both trypsin fragments of VP4 (VP5* and VP8*) are capable of mediating passive protective immunity in a mouse model of rotavirus infection (26, 32). In addition, genetic studies have also shown that antibodies to either VP4 or VP7 can mediate passive protection in a murine model (33). The exact role of antibodies to VP4 and VP7 as well as cellular immunity in mediating active protection in vivo has not yet been well established.

Adopting an approach similar to the one used by Mosser et al. (30), we were also able to characterize two groups of MAbs to RRV on the basis of their neutralization potency. Antibodies directed to the surface glycoprotein VP7 shared a potent neutralizing characteristic in that, upon incubation with the virus, the infectious titer of virus dropped steeply by three or more orders of magnitude. Antibodies directed at the A (159 and 4F8) and C (5H3) epitopes of VP7 each displayed this characteristic, which seems logical since both the A and C epitopes on VP7 appear to form a single large neutralization domain (8, 26). On the other hand, each of the five MAbs directed at VP4 appeared to be weakly neutralizing. The corresponding fraction of persistent infectivity in the presence of excess VP4 antibody was at least 100-fold higher than that observed for the VP7 MAbs (compare Fig. 1A and B). Despite this difference, the concentration of antibody at which significant neutralization could be first detected was comparable for both groups of antibodies. In addition, in vivo the protective effect of antibodies to VP4 and VP7 could not be differentiated (32, 33).

The gradual slope of the neutralization curves obtained with the VP4 MAbs compared with the VP7 curves makes unlikely the possibility of a single-hit kinetic interaction in the neutralization of RRV by these MAbs. Since there are roughly 10 times as many VP7 molecules as VP4 molecules per virion, the difference in these slopes is even more significant. In fact, assuming that the number of VP4 molecules per virion is 120 (36, 49) and that only 1 of 100 to 1 of 1,000 virions is infectious in cell cultures, it follows that in the conditions of our assays, the number of VP4 epitopes available can be estimated to be between 10^{12} to 10^{13}/ml versus 10^{13} to 10^{14} VP7 epitopes. At antibody concentrations yielding 90% neutralization, approximately 4×10^{13} VP4-specific antibody molecules were present in the reaction mixture, which means an antibody-to-epitope ratio of at least 4:1. This suggests that interaction of VP4 antibody with most, if not all, VP4 molecules on the virion is required for infectivity to be abolished. On the other hand, as few as 10^{12} VP7-specific antibodies were required to induce a comparable effect. Thus, binding of VP7-specific antibodies to 1 to 10% of the corresponding antigenic molecules on the virion appears to be sufficient for neutralization to take place.

It seems reasonable to assume that an antibody that neutralized by blocking viral attachment would have to bind to most if not all of the viral attachment proteins, while antibodies that neutralized at concentrations well below virion saturation probably neutralize by mechanisms other than inhibition of binding. The results of the cell binding inhibition experiments (Fig. 2A and B) support the hypothesis that VP8* MAbs neutralize by blocking RRV binding, since four of the five VP4 MAbs appeared to significantly reduce the extent of virus attachment to MA104 cells. It is of particular interest that all the MAbs specific for the VP8* subfragment of VP4 showed this effect, whereas MAb 2G4, which recognizes the VP5* fragment of the same protein, did not. In addition, the antibodies to VP7, with the exception of 57.8, did not hinder viral binding to the cell monolayer. The comparable extent to which these VP8* antibodies mediate neutralization and block virus attachment, as well as the similar range of concentrations at which they exert both effects, suggests that these MAbs indeed impair virus replication by preventing anchoring of virions to the cell surface. Consistent with this hypothesis is the growing evidence which indicates that VP4 is a likely candidate for the cell attachment protein of rotavirus. Genetic and biochemical studies have demonstrated that VP4 is the rotavirus hemagglutinin (11, 24). Recent studies (12a) indicate that hemagglutinin activity is localized to VP8*. Cryoelectron microscopic analysis of rotavirus has demonstrated that VP4 forms a spikelike structure which extends beyond the viral surface by over 10.0 nm, making it resemble other viral cell attachment structures (36, 37, 49). In contrast, some researchers have proposed that the VP7 protein is responsible for bovine and human rotavirus anchoring to the cell (13, 28, 38). In some studies, a protein from virus-infected cell lysates with a molecular weight corresponding to that of VP7 was observed to bind to MA104 cell monolayers. The identity of this putative cell-binding protein and the relevance of this phenomenon to binding of virions to cells have recently been questioned by Bass and colleagues (1), who concluded that a nonstructural protein (NS35), and not VP7, was the peptide with cell attachment characteristics found in infected cell lysates. If VP4 is the cell attachment protein, it seems logical that antibodies to this protein would efficiently inhibit viral binding to cells. Prior studies using hyperimmune sera (28, 39) as well as our own study indicate that some VP7-specific antibodies can also efficiently inhibit binding of virus to cells. Since VP7 and VP4 are closely juxtaposed on the viral surface and since antibodies to VP7 can clearly inhibit hemagglutinin by an indirect steric effect, this finding is not surprising. Since MAb 57.8 is an IgM antibody, this reagent would be the VP7 MAb most likely to inhibit virus-cell interaction on the basis of an indirect steric effect. However, three MAbs directed at the major neutralization domain on VP7 were very inefficient at inhibiting rotavirus binding, making it unlikely that this protein is the primary cell attachment structure.

It is of interest that papain treatment of RRV neutralized with the three VP7 MAbs belonging to the IgG class (159, 4F8, 5H3) led to substantial recovery of infectivity. On the

other hand, in situ-generated Fab fragments from the MAbs directed at VP8* neutralized RRV to an extent comparable to that of their corresponding divalent molecules. The finding that monovalent binding of VP8* antibodies is sufficient for neutralization further supports the hypothesis that these MAbs exert their effect by inhibiting RRV binding to cells. It also suggests that the cell receptor-binding domain on the virion is located within or near the binding region of the Fab fragment on VP8*. Complexing the Fab fragments bound to VP8* with anti-IgG antibodies did not result in any significant increase in neutralization activity as would be expected if these molecules functioned by inhibiting binding of virus to the cell. On the other hand, such antiglobulin treatment of Fab fragments from the VP7 MAbs led to full restoration of neutralization. Using a similar approach, Icenogle et al. (19) and Emini and co-workers (10) concluded that neutralization of poliovirus by selected MAbs requires bivalent binding of the antibody molecule. Accordingly, bivalent binding might be required for rotavirus neutralization by antibodies specific for VP7. Because of the saturating amount of anti-IgG antibody used in our experiments, it is unlikely that the neutralization observed upon addition of anti-IgG antiserum simply reflects aggregation of otherwise infectious virus-Fab complexes. Moreover, a similar finding was not observed for the VP8*-specific MAbs.

The observation that the VP8*-specific MAbs could neutralize cell-bound RRV, even if slightly less efficiently and at higher antibody concentrations than in experiments with virus in solution, initially appeared to contradict the hypothesis that these antibodies functioned by inhibiting viral attachment to the cell. However, we were able to demonstrate that the same VP8* MAbs that neutralize cell-bound virus can also mediate a significant release of previously bound virions from the cell surface. Antibodies to VP5* and VP7 were less efficient at eluting prebound rotavirus despite the fact that they efficiently neutralized this virus. Presumably, the binding of the viral attachment protein to the cell surface receptor and VP8* antibody to the cell attachment site on the virion is a dynamic process. The affinity for the antibody-virus interaction may be higher than the affinity of the interaction between virus and the cell surface. In agreement with this hypothesis is the finding by Colonno et al. (6) that an MAb specific for the rhinovirus receptor on HeLa cells was able to physically displace virions from the membrane-virus complex. If antibodies are directed specifically at the cellular attachment site on the virus, they might be expected to elute bound virions in the same manner that antibodies directed at the viral receptor elute virus.

Most of the VP7- and VP5*-specific MAbs tested did not elute cell-bound virus, although these antibodies could efficiently neutralize virions adsorbed to cells. The only exception to this observation was the VP7 MAb 4F8. However, this MAb mediated release of virus to an extent about 100-fold less than its neutralizing activity under similar experimental conditions. Altogether, these data suggest that VP7- and VP5*-directed antibodies interfere with postbinding steps in the virus replication cycle. The exact mechanism by which antibodies directed at VP7 neutralize virus is unknown and must await further study.

With respect to the VP5* MAb 2G4, it might be speculated that this antibody blocks productive penetration of RRV into the cell. In a previous study (21), virus neutralized by 2G4 was shown not to mediate ^{51}Cr release from cells. This observation was interpreted as indicating that the neutralized virions did not enter MA104 cells by a direct plasma membrane penetration pathway (21). Other studies (24) demonstrated that the site at which the 2G4 MAb selects escape mutants has sequence similarity to a putative membrane fusion region. Finally, Prasad and colleagues (36), using 2G4 Fab fragments and cryoelectron microscopy, have localized the epitope for this antibody to a region close to, but not at, the distal extremity of the VP4 spike of SA11. They speculated that the spike tip might be responsible for virus attachment and that the region recognized by 2G4 might be involved on viral entry. 2G4 Fab fragments were the only VP4-directed MAb Fabs which underwent neutralization enhancement when treated with antiglobulin. The reason for this specific effect is not clear and requires further study.

Although the mechanisms of virus neutralization have been studied for many years, there remains much that is not understood concerning this complex process. Certain fundamental methodologic problems remain to be solved. Most studies, including our own, must use high concentrations of virus that are not likely to occur during natural infection. Studies that monitor radiolabeled virions are always subject to potential artifact since the PFU/particle ratio is frequently very low. This is probably the case for RRV. However, several findings in this study make it likely that the data observed are biologically relevant. First, the observation that all MAbs investigated were able to neutralize RRV after its binding to the cell appears to rule out the possibility that antibody-mediated viral aggregation is responsible for the neutralization activity of these antibodies. Second, the finding that neutralizing antibodies to different surface proteins (VP4 and VP7) and different regions of VP4 behaved distinctly indicates that the ability of VP8* MAbs to block binding is not a universal property of antibodies directed at the viral surface. Prior studies had demonstrated that differences between the MAbs under study could not be explained by differences in affinity (42). Finally, several distinct experiments including neutralization curves, binding inhibition assays, elution assays, and protease digestion studies all gave results compatible with the hypothesis that antibodies directed at VP8*, but not at VP5* or VP7, neutralize RRV by inhibiting viral binding.

Our data suggest that antibodies directed to different domains of rotavirus surface proteins neutralize virus in vitro by different mechanisms. In particular, antibodies to VP8* seem to interfere with the process of virus binding to its receptor on the cell membrane. In the experimental conditions used in this study, the VP4 antibodies do not cause as dramatic a decrease in infectivity as that shown for the VP7 MAbs. It is noteworthy, however, that at least one of the VP8* and one of the VP5* MAbs studied here were previously demonstrated to protect infant mice from challenge with infectious virus (27, 33). Further studies will be required to establish whether the proposed mechanism of action for VP8* antibodies in vitro may be responsible for the protective activity of these antibodies in vivo. Additional studies will also be required to determine at what stage after virus binding antibodies to VP5* and VP7 mediate their neutralizing activity.

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REFERENCES

1. **Bass, D. M., E. R. Mackow, and H. B. Greenberg.** 1990. NS35 and not vp7 is the soluble rotavirus protein which binds to target cells. J. Virol. **64**:322–330.
2. **Benfield, D. A., E. A. Nelson, and Y. Hoshino.** 1987. A monoclonal antibody to the Gottfried strain of porcine rotavirus which neutralizes rotavirus serotypes 3, 4 and 6, p. 111. Abstracts of the Seventh International Congress of Virology. National Research Council Canada, Ottawa, Ontario, Canada.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Brioen, P., D. Dekegel, and A. Boeyé.** 1983. Neutralization of poliovirus by antibody-mediated polymerization. Virology **127**: 463–468.
5. **Carthew, P.** 1976. The surface nature of proteins of a bovine enterovirus, before and after neutralization. J. Gen. Virol. **32**:17–23.
6. **Colonno, R. J., P. L. Callahan, and W. J. Long.** 1986. Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinovirus. J. Virol. **57**:7–12.
7. **Dimmock, N. J.** 1984. Mechanisms of neutralization of animal viruses. J. Gen. Virol. **65**:1015–1022.
8. **Dyall-Smith, M. L., I. Lazdins, G. W. Tregear, and I. H. Holmes.** 1986. Location of the major antigenic sites involved in rotavirus serotype-specific neutralization. Proc. Natl. Acad. Sci. USA **83**:3465–3468.
9. **Emini, E. A., A. J. Dorner, L. F. Dorner, B. A. Jameson, and E. Wimmer.** 1983. Identification of a poliovirus neutralization epitope through use of neutralizing antiserum raised against a purified viral structural protein. Virology **124**:144–151.
10. **Emini, E. A., P. Ostapchuk, and E. Wimmer.** 1983. Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. J. Virol. **48**:547–550.
11. **Estes, M. K.** 1990. Rotaviruses and their replication, p. 1329–1351. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
12. **Estes, M. K., D. Y. Graham, and B. B. Mason.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. **39**:879–888.

12a. **Fiore, L., H. B. Greenberg, and E. R. Mackow.** The VP8 fragment of VP4 is the rhesus rotavirus hemagglutinin. Virology, in press.

13. **Fukuhara, N., O. Yoshie, S. Kitakoa, and T. Konno.** 1988. Role of VP3 in human rotavirus internalization after target cell attachment via VP7. J. Virol. **62**:2209–2218.
14. **Fuller, A. O., R. E. Santos, and P. G. Spear.** 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit virus attachment to cells but prevent penetration. J. Virol. **63**:3435–3443.

14a. **Greenberg, H. B.** Unpublished data.

15. **Greenberg, H. B., J. Flores, A. R. Kalica, R. G. Wyatt, and R. Jones.** 1983. Gene coding assignments for growth restriction, neutralization, and subgroup specificities of the Wa and Ds-1 strains of rotavirus. J. Gen. Virol. **64**:313–320.
16. **Ho, D. D., J. C. Kaplan, I. E. Rackauskas, and M. E. Gurney.** 1988. Second conserved domain of gp120 is important for HIV infectivity and antibody neutralization. Science **239**:1021–1023.
17. **Homsy, J., M. Meyer, M. Tateno, S. Clarkson, and J. A. Levy.** 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science **244**:1357–1360.
18. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flors, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82**:8701–8704.
19. **Icenogle, J., H. Shiwen, G. Duke, S. Gilbert, R. Rueckert, and J. Anderegg.** 1983. Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. Virology **127**:412–425.
20. **Joklik, W. K.** 1964. The intracellular fate of rabbitpox virus rendered non-infectious by various reagents. Virology **22**:620–633.
21. **Kaljot, K. T., R. D. Shaw, D. H. Rubin, and H. B. Greenberg.** 1988. Infectious rotavirus enters cells by direct membrane penetration, not by endocytosis. J. Virol. **62**:1136–1144.
22. **Kapikian, A. Z., and R. M. Chanock.** 1990. Rotaviruses, p. 1353–1404. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
23. **Lee, P. W. K., E. C. Hayes, and W. K. Joklik.** 1981. Protein Sigma1 is the reovirus cell attachment protein. Virology **108**: 156–163.
24. **Mackow, E. R., J. W. Barnett, H. Chan, and H. B. Greenberg.** 1989. The rhesus rotavirus outer capsid protein VP4 functions as a hemagglutinin and is antigenically conserved when expressed by a baculovirus recombinant. J. Virol. **63**:1661–1668.
25. **Mandel, B.** 1976. Neutralization of poliovirus: a hypothesis to explain the mechanism and the one-hit character of the neutralization reaction. Virology **69**:500–510.
26. **Matsui, S. M., E. R. Mackow, and H. B. Greenberg.** 1989. Molecular determinant of rotavirus neutralization and protection. Adv. Virus Res. **36**:181–214.
27. **Matsui, S. M., P. A. Offit, P. T. Vo, E. R. Mackow, D. A. Benfield, R. D. Shaw, L. Padilla-Noriega, and H. B. Greenberg.** 1989. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralizing domain of VP7 and the VP8 fragment of VP4. J. Clin. Microbiol. **27**:780–782.
28. **Matsuno, S., and S. Inouye.** 1983. Purification of an outer capsid glycoprotein of neonatal calf diarrhea rotavirus and preparation of its antisera. Infect. Immun. **39**:155–158.
29. **Miller, N., and L. M. Hutt-Fletcher.** 1988. A monoclonal antibody to glycoprotein gp85 inhibits fusion but not attachment of Epstein-Barr virus. J. Virol. **62**:2366–2372.
30. **Mosser, A. G., D. M. Leippe, and R. R. Rueckert.** 1988. Neutralization of picornaviruses: support for the pentamer bridging hypothesis, p. 345–365. *In* B. L. Semler and E. Ehrenfeld (ed.), Molecular aspects of picornavirus infection and detection. American Society for Microbiology, Washington, D.C.
31. **Offit, P. A., G. Blavat, H. B. Greenberg, and H. F. Clark.** 1986. Molecular basis of rotavirus virulence: role of gene segment 4. J. Virol. **57**:46–49.
32. **Offit, P., R. D. Shaw, and H. B. Greenberg.** 1986. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins VP3 and VP7. J. Virol. **58**:700–703.
33. **Offit, P. A., H. F. Clark, G. Blavat, and H. B. Greenberg.** 1986. Reassortant rotaviruses containing structural proteins VP3 and VP7 from different parents induce antibodies protective against each parental serotype. J. Virol. **60**:491–496.
34. **Outlaw, M. C., and N. J. Dimmock.** 1990. Mechanisms of neutralization of influenza virus on mouse tracheal epithelial cells by mouse monoclonal polymeric IgA and polyclonal IgM directed against the viral haemagglutinin. J. Gen. Virol. **71**:69–76.
35. **Possee, R. D., G. C. Schild, and N. J. Dimmock.** 1982. Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus in vivo by inhibiting virion transcriptase activity. J. Gen. Virol. **58**:373–386.
36. **Prasad, B. V., J. W. Burns, E. Marietta, M. K. Estes, and W. Chiu.** 1990. Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. Nature (London) **343**:476–479.
37. **Prasad, B. V., G. J. Wang, J. P. Clerx, and W. Chiu.** 1988. Three-dimensional structure of rotavirus. J. Mol. Biol. **199**:269–275.
38. **Sabara, M., A. Barrington, and L. A. Babiuk.** 1985. Immunogenicity of a bovine rotavirus glycoprotein fragment. J. Virol. **56**:1037–1040.
39. **Sabara, M., J. Gilchrist, G. R. Hudson, and L. A. Babiuk.** 1985. Preliminary characterization of an epitope involved in neutralization and cell attachment of an epitope that is located on the major bovine rotavirus glycoprotein. J. Virol. **53**:58–66.
40. **Schlegel, R., and M. Wade.** 1983. Neutralized vesicular stomatitis virus binds to host cells by a different "receptor." Bio-

chem. Biophys. Res. Commun. **114**:774–778.

41. **Shaw, R. D., K. J. Fong, G. A. Losonsky, M. M. Levine, Y. Maldonado, R. Yolken, J. Flores, A. Z. Kapikian, P. T. Vo, and H. B. Greenberg.** 1987. Epitope-specific immune responses to rotavirus vaccination. Gastroenterology **93**:941–950.
42. **Shaw, R. D., P. T. Vo, P. A. Offit, B. S. Coulson, and H. B. Greenberg.** 1986. Antigenic mapping of the surface proteins of rhesus rotavirus. Virology **155**:434–451.
43. **Skinner, M. A., A. J. Langlois, C. B. McDanal, J. S. McDougal, D. P. Bolognesi, and T. J. Matthews.** 1988. Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. J. Virol. **62**:4195–4200.
44. **Svensson, L., H. Sheshberadaran, T. Vesikari, E. Norrby, and G. Wadell.** 1987. Immune response to rotavirus polypeptides after vaccination with heterologous rotavirus vaccines (RIT 4237, RRV-1). J. Gen. Virol. **68**:643–651.
45. **Taniguchi, K., S. Urasawa, and T. Urasawa.** 1984. Preparation and characterization of neutralizing monoclonal antibodies with different reactivity patterns to human rotaviruses. J. Gen. Virol. **66**:1045–1053.
46. **Thomas, A. A. M., P. Brioen, and A. Boeyé.** 1985. A monoclonal antibody that neutralizes poliovirus by cross-linking virions. J. Virol. **54**:7–13.
47. **Wohlfart, C.** 1988. Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms. J. Virol. **62**:2321–2328.
48. **Wohlfart, C. E. G., U. K. Svensson, and E. Everitt.** 1985. Interaction between HeLa cells and adenovirus type 2 virions neutralized by different antisera. J. Virol. **56**:896–903.
49. **Yeager, M., K. A. Dryden, N. H. Olson, H. B. Greenberg, and T. S. Baker.** 1990. Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. J. Cell Biol. **110**:2133–2144.